STUDIES ON A POSSIBLE PHOSPHORYL-ENZYME INTERMEDIATE IN THE CATALYTIC REACTION OF YEAST PHOSPHOGLYCERATE KINASE

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Received February 8, 1974

SUMMARY. A phosphoryl-enzyme intermediate as part of the mechanism of phosphoglycerate kinase has been suggested for the rabbit muscle enzyme (6) and the yeast enzyme (7,8). ATP in the binary enzyme-substrate complexes appeared to phosphorylate these enzymes and ADP-ATP exchange activities were observed (6,7,8). The present report shows, however, that highly purified yeast enzyme cannot be phosphorylated by ATP. On the other hand ADP-ATP exchange activity was obtained but this was proportional to trace amounts of adenylate kinase activity, which was found to contaminate the enzyme preparations. Thus a Ping Pong mechanism as an alternative to a mechanism including a ternary complex between the enzyme and its two substrates appears very improbable. Whether the enzyme or the phosphoryl-group-accepting substrate is responsible for the primary nucleophilic attack occurring in the ternary complex is still an open question, however. Yeast phosphoglycerate kinase appears to have no ATPase activity.

Steady state kinetic studies (1,2) on the reaction catalyzed by yeast phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) suggest that this enzyme, like many other phosphotransferases (cf. (3) for a review), reacts by a mechanism that includes a ternary complex between the enzyme and its two substrates. The bond cleaved during the catalytic reaction is between the oxygen and phosphorous atoms (4,5). Evidence for a phosphorylated enzyme as part of the catalytic reaction of the rabbit muscle enzyme has been presented (6). This evidence served as a stimulus for the present study. After completion of our work results suggesting a phosphorylated enzyme as part of the catalytic reaction of the yeast enzyme have been published by others (7,8). Our studies indicate that their results must be given a different interpretation.

MATERIALS AND METHODS

Enzymes. Phosphoglycerate kinase was prepared from baker's yeast (9) and the main electrophoretic component B (cf. (10)) was used. Glyceraldehyde-

phosphate dehydrogenase (EC 1.2.1.12) and pyruvate kinase (EC 2.7.1.40) from rabbit muscle, and lactate dehydrogenase (EC 1.1.1.27) from beef heart were products of Sigma Chemical Co.

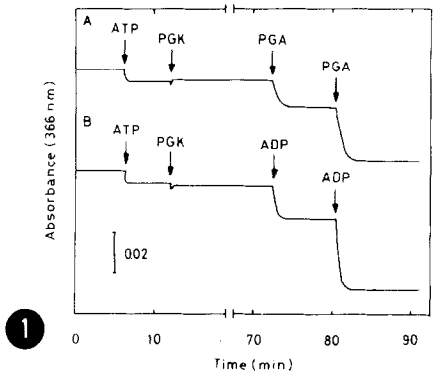
Reagents. [Base-U-^{14}C] adenosine 5'-diphosphate in aqueous solution was obtained from The Radiochemical Center, Amersham, England.

Activity measurements. The activity of phosphoglycerate kinase was measured by the spectrophotometric method of Bücher (11) at conditions described earlier (10,12). The activity of adenylate kinase was measured spectrophotometrically in the direction of ADP formation according to Chiu et al. (13). The assay mixture (1 ml) contained 1.0 mM ATP, 1.0 mM AMP, 3.0 mM $MgCl_2$, 1.0 mM phosphoenolpyruvate, 0.5 mM NADH, 0.25 M KCl, 50 mM Tris-HCl buffer (pH 7.80, 25°C), 32 activity units of pyruvate kinase, and 67 of lactate dehydrogenase. As in the phosphoglycerate kinase assay the change of NADH concentration was followed at 366 nm and the activity was expressed as $v = (dA_{366}/dt)_{t=0}$.

Spectrophotometric tests for phosphorylation of phosphoglycerate kinase by ATP. The experimental conditions were as described for the adenylate kinase assay (cf. above) but ATP and AMP were excluded (see Fig. 1).

Measurements of the exchange of [^{14}C] ADP with ATP. The reaction mixture containing 1.0 mM ATP, 1.0 mM ADP, 0.045 mM [^{14}C] ADP (40 % as [^{14}C] AMP) giving 1.2 x 10^6 cpm, 5.0 mM $MgCl_2$, and 50 mM Tris-HCl buffer (pH 7.80, 25°C) was supplied with varying amounts of phosphoglycerate kinase to a total volume of 100 µl. The experiments were performed at 25°C. Aliquots (5 µl) were withdrawn at appropriate time intervals and were applied to a plate for thin layer chromatography (cf. below). Each spot was scraped together and put into 10 ml of Insta-Gel scintillation solution (diluted with 0.6 volumes of xylene) for radioactivity measurements.

Thin layer chromatography. The nucleotides ATP, ADP, and AMP were separated by thin layer chromatography on DEAE cellulose according to Randerath (14). The best experimental conditions appeared when celluloses from Merck



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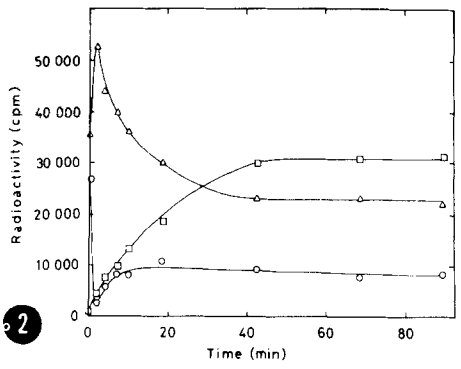

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Fig. 1. Lack of phosphorylation of phosphoglycerate kinase by ATP. At the times indicated the assay mixture (see MATERIALS AND METHODS) was supplied with 25 µl ATP (10 mM) and 50 µl phosphoglycerate kinase (PGK, 9.1 mg/ml). To test the sensitivity of the assay system successive additions of (A) 10 µl and 25 µl of 3-phospho-D-glycerate (PGA, 0.36 mM) and (B) 10 µl and 25 µl of ADP (0.50 mM) were performed. Corrections for the blank values were made.

Fig. 2. Distribution of radioactivity during the run of an ADP-ATP exchange experiment, performed with 37 µg of phosphoglycerate kinase. The activity of adenylate kinase was 0.013 % of the phosphoglycerate kinase activity. (O—O), ATP; (△—△), ADP; (□—□), ATP.

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and Whatman were mixed in equal proportions. The chromatogram was developed in 0.04 M HCl for about 3 hours, after which the spots were detected in ultraviolet light. R_f values were for ATP 0.06, ADP 0.34, and AMP 0.92.

RESULTS AND DISCUSSION

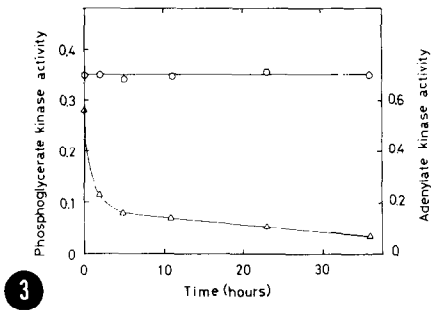
Spectrophotometric tests for phosphorylation of phosphoglycerate kinase.

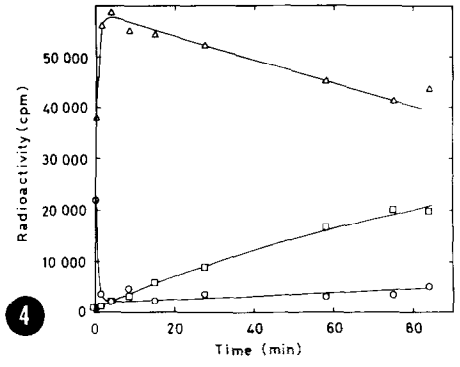
Any phosphorylation of the enzyme by ATP should be accompanied by the formation of ADP. Reaction mixtures containing ATP in large molar excess of phosphoglycerate kinase (230-640 µg/ml) were assayed for ADP formation during one hour. No change of absorbance was noticed (see Fig. 1). Judged from the results of Hass et al. (15) the assay system appeared sensitive enough for this test. That was further shown by repeated additions of proper amounts of 3-phospho-D-glycerate and ADP, respectively. These reagents appeared to cause the expected reactions to go to completion within some minutes (Fig. 1). The incorporation of one phosphoryl group per enzyme molecule should at the experimental conditions of Fig. 1 have caused a change of absorbance of 0.030 (the molecular weight of 45 000 for the enzyme (10) and the numeric value of

3 300 cm^{-1} for the difference in molar absorption of NADH and NAD^+ at 366 nm (16) were used in these estimations). Possible ATPase (EC 3.6.1.4) activity should cause this change to be much larger. The present results thus indicate that phosphoglycerate kinase is devoid of ATPase activity.

Exchange of $[^{14}C]$ ADP with ATP. Fig. 2 shows incorporation of radioactivity into ATP catalyzed by a phosphoglycerate kinase preparation. Initially there was also a rapid incorporation of radioactivity from AMP into ADP and the dominating reaction appeared to be $[^{14}C]$ AMP + ATP $\rightarrow$ $[^{14}C]$ ADP + ADP. Note that at zero time the specific radioactivity of AMP was high and that ATP was in large molar excess of AMP. As the time passed the reverse reaction continuously appeared to take place, causing incorporation of isotope into ATP. Equilibrium was reached after 45 min. The equilibrium constant, $K_{eq} = [AMP][ATP] / [ADP]^2$, as estimated from this experiment, is 0.52. This value is in good agreement with the constant determined earlier (17) for the adenylate kinase reaction. Tests on our phosphoglycerate kinase preparations showed that these contain traces of adenylate kinase activity (cf. below). At 40°C this additional activity decreased gradually (Fig. 3). After 36 hours at 40°C only 12 % of the adenylate kinase activity remained but the phosphoglycerate kinase activity was unaffected. The ADP-ATP exchange experiment was repeated with the heat-treated enzyme and the results are presented in Fig. 4, which shows that incorporation of radioactivity into ATP occurred at a much slower rate than previously (Fig. 2). There was still a rapid flow of radioactivity from AMP to ADP. The rate of this reaction was too rapid to be quantified, however. The reactions presented in Fig. 4 were far from equilibrium even after 85 min. The results with the heat-treated enzyme strongly indicate that the isotope exchange between ADP and ATP was caused by the adenylate kinase reaction.

Is phosphoglycerate kinase responsible for the adenylate kinase activity? Five different phosphoglycerate kinase preparations were tested for adenylate kinase activity. This was shown to vary between 0.001 and 0.013 % of the phos-





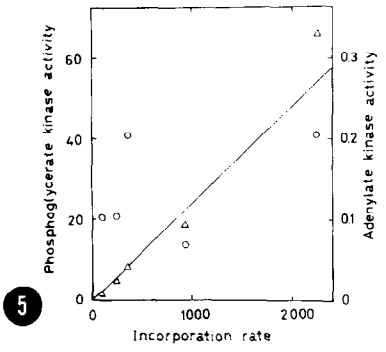


Fig. 3. The time-dependences of the activities of phosphoglycerate kinase (in min^{-1}) (O—O) and adenylate kinase (in $hour^{-1}$) (Δ—Δ) during treatment at 40°C, pH 8.2. The protein concentration was 6.36 mg/ml. In the activity measurements the enzyme solution was diluted 20 000 and 100 times, respectively.

Fig. 4. Distribution of radioactivity during the run of an ADP-ATP exchange experiment, performed with 57 μg of phosphoglycerate kinase treated at 40°C for 36 hours (cf. Fig. 3). The activity of adenylate kinase was 0.002 % of the phosphoglycerate kinase activity. (O—O), AMP; (Δ—Δ), ADP; (□—□), ATP.

Fig. 5. The activities of phosphoglycerate kinase (in min^{-1}) (O—O) and adenylate kinase (in $hour^{-1}$) (Δ—Δ) versus the initial rate of incorporation of radioactivity into ATP, expressed as $v = (d(cpm)/dt)_{t=0}$ (in min^{-1}). The specific activity of phosphoglycerate kinase was the same in all the preparations used.

phoglycerate kinase activity, if expressed in comparable units. The rates of ^{14}C-exchange between ADP and ATP catalyzed by the different preparations were determined. Fig. 5 shows the activities of phosphoglycerate kinase and adenylate kinase, respectively, versus the initial incorporation rate of radioactivity into ATP. The rate of incorporation increased with the

adenylate kinase activity. No correlation of this rate with the phosphoglycerate kinase activity can be seen.

Conclusions and comments. The present results clearly indicate that ATP in the binary enzyme-substrate complex is not able to phosphorylate yeast phosphoglycerate kinase. Earlier results (6,8) suggested that only about 35 % of the enzyme molecules could be phosphorylated and that the phosphorylation required phosphoenolpyruvate and pyruvate kinase to trap the ADP formed. This was not shown directly, for example by following the pyruvate kinase reaction. Fig. 1 shows that such a test is sensitive enough easily to detect even a partial phosphorylation. The earlier used criterion for phosphorylation was appearance of ^{32}P in the enzyme peak after gel filtration on Sephadex G-25 (6,8). Incorporation of ^{32}P into any impurity having a molecular weight $>$ 5 000 would give the same result, however. The purity of the rabbit muscle enzyme, used in the above experiments, has been questioned (18). The specific activity of the yeast enzyme used was low also, 500-700 U/mg at 25°C instead of a value close to 1000 for a highly purified enzyme (10,18). Note that pyruvate kinase in the presence of phosphoenolpyruvate and ATP has been shown to cause phosphorylation of something unknown (19). Apparent phosphorylation caused by artifacts has elegantly been demonstrated and discussed by Hass et al. (15). Results of Bücher (11) indicate that yeast phosphoglycerate kinase has a fairly high affinity for pyrophosphate.

Despite all precautions earlier taken (7) to eliminate contaminating enzymes in the yeast phosphoglycerate kinase preparations adenylate kinase appears not to have been excluded as a possible catalyst of the ADP-ATP exchange reaction for the following three reasons: 1. This enzyme has, like phosphoglycerate kinase (20), no thiol group essential for the catalytic activity (13). 2. Diethylpyrocarbonate, which effectively inactivates for example nucleosidediphosphate kinase (EC 2.7.4.6) (7), has been shown to cause a small decrease in the phosphoglycerate kinase activity and a concomitant loss in the rate of the isotope exchange has been observed (7). Treatment

of our enzyme preparations with diethylpyrocarbonate, at conditions earlier used by others (7,8), caused a decrease of the activities of both phosphoglycerate kinase and adenylate kinase concurrently, about 30 % at 24°C (unpublished observations). 3. Various nucleoside triphosphates can to different extents replace ATP as a substrate of adenylate kinase (21). Thus, it seems possible that the earlier observed effects (7) of nucleoside triphosphates on the rate of the ADP-ATP exchange were caused by effects on the adenylate kinase reaction.

Walsh and Spector have endeavoured to produce a rabbit muscle enzyme free from adenylate kinase (myokinase). It is, however, impossible from their paper (6) to appraise how they succeeded. The rates of the partial ADP-ATP exchange observed in our study (apparently caused by adenylate kinase activity being 0.001-0.013 % of the phosphoglycerate kinase activity) are of the same order of magnitude as those reported for the commercial yeast enzyme (7) and the rabbit muscle enzyme (6). As yeast phosphoglycerate kinase appears to lack ADP-ATP exchange activity a Ping Pong mechanism (22) as an alternative to the earlier suggested ternary complex mechanism (1,2) can be excluded. At present it cannot be settled, however, whether the enzyme or the phosphoryl-group-accepting substrate is responsible for the primary nucleophilic attack, occurring as an intermediary step in the ternary complex between the enzyme and its two substrates.

ACKNOWLEDGEMENTS. We wish to thank Professor Bo G. Malmström for continuous support of this work and Mrs. Ingrid Nüth for excellent technical assistance. We are also grateful to Professor Ulf Lagerkvist for placing scintillation spectrometer at our disposal. This work was supported by research grants from the Swedish Natural Science Research Council.

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